Diversity of Dissimilatory Bisulfite Reductase Genes of Bacteria Associated with the Deep-Sea Hydrothermal Vent Polychaete Annelid Alvinella pompejana

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A unique community of bacteria colonizes the dorsal integument of the polychaete annelid Alvinella pompejana, which inhabits the high-temperature environments of active deep-sea hydrothermal vents along the East Pacific Rise. The composition of this bacterial community was characterized in previous studies by using a 16S rRNA gene clone library and in situ hybridization with oligonucleotide probes. In the present study, a pair of PCR primers (P94-F and P93-R) were used to amplify a segment of the dissimilatory bisulfite reductase gene from DNA isolated from the community of bacteria associated with A. pompejana. The goal was to assess the presence and diversity of bacteria with the capacity to use sulfate as a terminal electron acceptor. A clone library of bisulfite reductase gene PCR products was constructed and characterized by restriction fragment and sequence analysis. Eleven clone families were identified. Two of the 11 clone families, SR1 and SR6, contained 82% of the clones. DNA sequence analysis of a clone from each family indicated that they are dissimilatory bisulfite reductase genes most similar to the dissimilatory bisulfite reductase genes of Desulfovibrio vulgaris, Desulfovibrio gigas, Desulfobacterium autotrophicum, and Desulfobacter latus. Similarities to the dissimilatory bisulfite reductases of Thermodesulfovibrio yellowstonii, the sulfide oxidizer Chromatium vinosum, the sulfur reducer Pyrococcus islandicus, and the archaeal sulfate reducer Archaeoglobus fulgidus were lower. Phylogenetic analysis separated the clone families into groups that probably represent two genera of previously uncharacterized sulfate-reducing bacteria. The presence of dissimilatory bisulfite reductase genes is consistent with recent temperature and chemical measurements that documented a lack of dissolved oxygen in dwelling tubes of the worm. The diversity of dissimilatory bisulfite reductase genes in the bacterial community on the back of the worm suggests a prominent role for anaerobic sulfate-reducing bacteria in the ecology of A. pompejana.

Alvinella pompejana is a polychaete annelid that inhabits high-temperature environments of active deep-sea hydrothermal vents along the East Pacific Rise (14). Colonies of this worm and a congener, Alvinella caudata, are found on the sides of black smoker chimneys, where hydrothermal fluids emit at temperatures near 350°C. The worms live in areas of steep chemical and thermal gradients where hydrothermal fluids move through the chimney wall and mix with the surrounding seawater (15, 16), creating a temperature gradient of maximally 60°C (9). The physiological and biochemical adaptations allowing the worm to thrive under extreme conditions not normally tolerated by eukaryotes are unknown.

The worm’s dorsal integument is covered by a diverse community of bacteria dominated by conspicuous filamentous members of the epsilon group of the class Proteobacteria (8, 18). Earlier culture efforts revealed a community composed of both aerobes and facultative anaerobes (25), sulfur oxidizers, sulfate reducers, nitrifiers, nitrate respirers, denitrifiers, and nitrogen fixers. Characteristics common to many of these isolates are resistance to cadmium, zinc, arsenate, and silver and tolerance of high concentrations of copper (21).

The role of epibiotic bacterial symbionts in the ecology of the host worm is not clear. A nutritional role analogous to the symbiotic associations between chemosynthetic bacteria and other invertebrate hosts (10, 11) has been proposed, but there is little evidence of chemosynthesis (CO₂ fixation) (2). It has also been suggested that the symbionts detoxify the worm’s immediate environment of metals and hydrogen sulfide (2).

Our primary goal is to understand the interaction between A. pompejana and its associated bacteria by identifying the abundant bacterial epi-bionts and their metabolic capacities. In this study, we explored the diversity of a gene involved in anaerobic sulfate respiration and was probably utilized by the worm’s environment contains abundant sulfur and little dissolved oxygen. Dissimilatory bisulfite reductase is the terminal redox enzyme that catalyzes the reduction of sulfite to sulfide during anaerobic respiratory sulfate reduction. Prokaryotic dissimilatory bisulfite reductases have an αβγ structure (4, 12, 24) and possess iron-sulfur clusters and siroheme prosthetic groups. This is the key enzyme involved in sulfate respiration and was probably utilized by the common ancestors of bacteria and archaea (31).

MATERIALS AND METHODS

Animal collection. Specimens of A. pompejana were collected from active vent sites designated 13°N (12°48′N, 103°56′W) and 9°N (9°50′N, 104°17′W) on the East Pacific Rise at a depth of approximately 2,620 m in November of 1994 and 1995. Animals were collected by the deep-submergence vehicle Alvin and held in an insulated container which maintained the collection at ~5°C until surfacing. Once on board, specimens were held at 2°C until they were sampled for bacteria and nucleic acids as described below.

DNA purification. Bacteria were aseptically removed from the dorsal surface of freshly collected A. pompejana for DNA purification. Forcesps cleaned with 70% ethanol were used to remove approximately 50-μl tufts of hair-like projections covered with bacteria. Bacteria were homogenized in 1 ml of 5 M guanidine thiocyanate–50 mM Tris-HCl (pH 7.4)–25 mM EDTA–0.8% 2-mercaptoethanol. A brief centrifugation was performed to remove the bulk of the mineral grains, and the homogenates were stored at ~80°C until DNA extraction was performed in the laboratory. Aliquots (100 μl) of the thawed homogenates were incubated for 1 h with 25 μl of 20% Chelex 100 (32) while being mixed on a rotating wheel.
Following a brief centrifugation to remove the Chelex 100, total nucleic acids were extracted with the IsoQuick nucleic acid extraction kit (ORCA Research, Inc., Bothel, Wash.). In accordance with the manufacturer’s instructions, the first extraction was performed at 65°C for 10 min, and the second extraction was done at room temperature. The nucleic acids were concentrated by isopropanol precipitation and quantified spectrophotometrically.

PCR. Deoxynucleoside triphosphates (dTTP, dCTP, dGTP, and dATP), 1.25 mM MgCl₂, approximately 7 ng of template DNA per reaction mixture, 1.0 U of Taq DNA polymerase (Promega) in a total reaction volume of 20 μl. The thermocycling was performed by using a RotoCycler gradient thermocycler (Stratagene, La Jolla, Calif.) with thermocycling conditions including 1.5 min of denaturation at 94°C, 2.5 min of primer annealing at 60°C, and 3 min of primer extension at 72°C. This cycle was repeated 30 times. A hot start (13°C) was performed by warming the reaction mixtures to 95°C before adding the primer DNA polymerase.

Clone library construction and screening. PCR products obtained from two A. pompejana specimens collected at 9°N and 13°N were cloned by using the TA Cloning kit with pCR II vector (Invitrogen, San Diego, Calif.) in accordance with the manufacturer’s instructions. Approximately 200 hundred recombinants were screened for full-size inserts (approximately 1.4 kb) by transferring small aliquots of DNA to PCR mixtures containing the bisulfite reductase primers and thermocycling under the same conditions described above. Colonies that did not produce amplification were eliminated from the library. The PCR products, which were all approximately 1.4 kb, were cut with the restriction endonuclease MboI. The restriction fragments were resolved by agarose gel electrophoresis with 3% NuSieve (FMC, Rockland, Maine) agarose. Clones with identical restriction patterns were grouped together into clone families.

Nucleotide sequencing. Nucleotide sequencing was performed with a Perkin-Elmer (Foster City, Calif.) ABI PRISM 310 genetic analyzer and an ABI PRISM dye termination cycle sequencing ready kit with Ampli Taq DNA polymerase in accordance with the manufacturer’s instructions. Double-stranded DNA templates were prepared according to the manufacturer’s alkaline-lysis and polyethylene glycol precipitation protocols. Sequencing primers M13 forward or reverse were used to sequence on from the cloning vector depending on the orientation of the cloned PCR product. The sequencing of complementary strands was performed with primers SFITE450AR (AGGCCCTGACGCTTCA TCAG) and SFITE471AR (TAAACCCTAGAAGGTTGAGGC) from clone families SR1, 2, 4, 5, 7, and 8 and SR3, 6, 9, 10, and 11, respectively. These primers bind to positions 450 and 471 nucleotides into the PCR products numbered with the clone representing SR2 as a reference. Approximately 500 nucleotides were sequenced for each strand. The sequences were initially analyzed by a search of all nonredundant GenBank CDS translations, PDB, SwissProt, and PIR databases by using the Blast function of Sequence Navigator version V. 1.0.1 (Perkin-Elmer) was and re- fined by eye. The phylogenetic analysis was performed with the SEQBOOT, PROTDIST, NEIGHBOR, and CONSENSUS programs in PHYLIP version 3.527.

RESULTS AND DISCUSSION

Occurrence of dissimilatory bisulfite reductase genes. We investigated the presence of dissimilatory bisulfite reductase genes in the microbial community on the dorsal surface of A. pompejana, because preliminary data suggested that this habitat is high temperature, anoxic, and rich in potential electron acceptors, e.g., sulfate (9, 17). Since the chemical and physical environment will dictate the specific physiological capability of the bacteria, it was logical to investigate genes involved in the anaerobic respiration of sulfate. We used primers previously shown to be very effective with known sulfate reducers from the genera Desulfovibrio, Desulfubalis, and Desulfobacter (22).

Genes encoding dissimilatory bisulfite reductase were detected in every DNA sample isolated from microbes on the dorsal surface of A. pompejana (Fig. 1). Amplifications were obtained from both the anterior and posterior dorsal surfaces of A. pompejana collected at both 13°N and 9°N on the East Pacific Rise. The amplicons were approximately the same size generated in control amplifications of the dissimilatory bisulfite reductase gene of D. vulgaris (1.4 kb) (22). On agarose gels, the amplification products typically appeared as two closely spaced bands. The higher-molecular-weight band was slightly larger than the 1.4-kb amplicon produced from D. vulgaris.

Clone library construction and characterization. A clone library of PCR products was used to explore the diversity of dissimilatory bisulfite reductase genes of the bacteria on the dorsal surface of A. pompejana. The library consisted of clones from two PCR products enriched in the high- and low-molecular-weight bands, respectively (Fig. 1). The library contained 154 clone fragments that were assembled into 11 clone families based on MboI restriction fragment banding patterns (Fig. 2). The library was dominated by clone families SR1 and SR6, which...
TABLE 1. Abundance of clone families in the library of bisulfite reductase gene fragments amplified by PCR from the microbial community associated with the dorsal surface of *A. pompejana*.

<table>
<thead>
<tr>
<th>Clone family</th>
<th>No. of clones</th>
<th>% of clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>SR1</td>
<td>68</td>
<td>44</td>
</tr>
<tr>
<td>SR2</td>
<td>1</td>
<td>0.65</td>
</tr>
<tr>
<td>SR3</td>
<td>8</td>
<td>5.2</td>
</tr>
<tr>
<td>SR4</td>
<td>2</td>
<td>1.3</td>
</tr>
<tr>
<td>SR5</td>
<td>8</td>
<td>5.2</td>
</tr>
<tr>
<td>SR6</td>
<td>59</td>
<td>38</td>
</tr>
<tr>
<td>SR7</td>
<td>4</td>
<td>2.5</td>
</tr>
<tr>
<td>SR8</td>
<td>1</td>
<td>0.65</td>
</tr>
<tr>
<td>SR9</td>
<td>1</td>
<td>0.65</td>
</tr>
<tr>
<td>SR10</td>
<td>1</td>
<td>0.65</td>
</tr>
<tr>
<td>SR11</td>
<td>1</td>
<td>0.65</td>
</tr>
</tbody>
</table>

Together accounted for 82% of the clones (Table 1). Clone families SR1 and SR6 were detected exclusively in libraries of the lower- and higher-molecular-weight PCR products, respectively. The balance of the library was composed of nine families obtained from both the high- and low-molecular-weight PCR products, which each contained 5% or fewer of the clones.

**Comparative sequence analysis of dissimilatory bisulfite reductase gene amplicons.** Comparative sequence analysis revealed high similarity between the clone families and the alpha subunits of dissimilatory bisulfite reductases. The BLASTP program (3) produced statistically significant alignments of the 11 clone families with every dissimilatory bisulfite reductase alpha subunit in the database (nonredundant GenBank CDS translations, PDB, SwissProt, SpPupdate, and PIR sequences [release date 6 May 1998]). For example, alignments of clone family SR1 had the smallest sum probabilities, ranging from $2.3 \times 10^{-58}$ to $8.6 \times 10^{-27}$. The alignment with the *D. vulgaris* alpha subunit contained 85 identical amino acids out of the 112 in the alignment and had the smallest probability of occurring by chance alone. The most significant alignment with a gene other than a dissimilatory bisulfite reductase was made with the polyferredoxin gene of *Methanococcus voltae* (smallest sum probability, 0.022) and likely occurred by chance alone.

The similarity of the 5’ end of the amplicons with the alpha subunits of dissimilatory bisulfite reductase genes was consistent with binding of primer P94-F to a region of the operon coding for the alpha subunit in *D. vulgaris* and *A. fulgidus* (22). However, a significant alignment with the beta subunit would not have been unexpected. The genes coding for the two subunits of dissimilatory bisulfite reductase evolved by the duplication of a common ancestral gene and therefore contain segments of statistically significant nucleotide and amino acid similarity (19).

The 11 clone families were most similar to the dissimilatory bisulfite reductases of bacterial sulfate reducers. A CLUSTAL alignment of the conceptual translations of the 11 clone families with the alpha subunits of the dissimilatory bisulfite reductases of *D. vulgaris*, *Desulfovibrio gigas*, *Desulfobacterium autotrophicum*, *Desulfobacter latus*, *Desulfotomaculum ruminis*, *Thermodesulfovibrio yellowstonii*, *Archeoglobus fulgidus*, *Chromatium vinosum*, and *Pyrobaculum islandicum* contained regions of high conservation interspersed with regions of variability (Fig. 3). The similarity (percent identical aligned amino acids) between the clone families and the bacterial sulfate reducers *D. vulgaris*, *D. gigas*, *D. autotrophicum*, and *D. latus* ranged from 60.4 to 75.0%. The similarities to *T. yellowstonii* (thermophilic bacterial sulfate reducer), *A. fulgidus* (an archaean sulfate reducer), and *C. vinosum* (a sulfur oxidizer) were lower (41.7 to 46.9%, 53.1 to 63.5%, and 44.8 to 47.9%, respectively). The clone families had very little similarity to an archaean sulfur reducer, *P. islandicum* (25.0 to 29.2% similar).

Inspection of the alignment revealed two groups consisting of clone families SR1, SR2, SR4, SR5, and SR8 and SR3, SR6, SR9, SR10, and SR11, respectively. Similarity within these two groups ranged from 87.5 to 100%, while similarity between members of these groups ranged from 62.5 to 68.7%. Similarities within the two groups were at the high end of this range (87.5 to 100%), and similarities between members of the two groups were lower (62.5 to 68.7%). Clone families that were identical at the amino acid level were 99.1 to 100% similar at the nucleotide level. For example, clone families SR10 and SR11 had identical nucleotide sequences over 287 bases at the 5’ end of the insert, but different restriction digest patterns of these two clone families indicated sequence variation elsewhere in the gene (Fig. 2).

A phylogenetic tree drawn with the neighbor-joining algorithm contained two clades of bisulfite reductase clone families supported by bootstrap values of 99 that were clearly separate from previously described sulfate-reducing bacteria (Fig. 4). Two *Desulfovibrio* species formed another well-supported clade (bootstrap value of 76) separate from the clade containing *D. latus* and *D. autotrophicum* (bootstrap value of 96). There is probably some relationship between the similarity of dissimilatory bisulfite reductase genes and conventional taxonomic descriptors (i.e., genus, species, etc.), because there is a high degree of similarity between the evolutionary relationships inferred from 16S rRNA and dissimilatory bisulfite reductase genes (31). The two clades comprised of clone families probably represent two previously uncharacterized genera of sulfate-reducing bacteria, because similarities between these two clades (62.5 to 68.7%) were lower than the similarities between species within the genus *Desulfovibrio* (79.2%).

Amino acid identity between sequences of the clone families and sequences reported by Wagner et al. (31) were typically less than 77%, supporting the idea that they represent previously uncharacterized sulfate-reducing bacteria. The partial sequences from these organisms were not included in the phylogenetic tree due to the small overlap with the region of the gene sequenced for the clone families. The high similarity between clone families within clades (greater than 87.5%) suggests that they are phylogenotypes more closely related than genera.

The clade containing *C. vinosum*, *P. islandicum*, *A. fulgidus*, and *T. yellowstonii* was not well supported in this analysis, because the amount of variation was not suited to the great evolutionary distances separating these genera. The dissimilatory bisulfite reductases of *P. islandicum*, *A. fulgidus*, and *C. vinosum* are true homologs (19) and seem to have evolved from a common ancestral protein that divided into three independent lineages prior to the divergence of archaea and bacteria (23). The highly conserved siroheme-binding region of the gene that revealed this relationship is outside the portion of the gene amplified by primers P94-F and P93-R used in this study.

The primers designed by Karkhoff-Schweizer et al. (22) used in this study may have produced a conservative measure of the diversity of genes for dissimilatory bisulfite reductase, because they were designed solely with the highly conserved regions of archaenal and bacterial genes. Modified versions of these primers based on the alignment of a larger number of sequences suggested by Wagner et al. (31) might reveal equal or greater diversity. Nevertheless, the diversity of dissimilatory bisulfite reductase genes we found suggests a role for anaerobic sulfate-reducing bacteria in the ecology of *A. pompejana*.

**Implications.** Sulfate-reducing bacteria are able to use a broad range of compounds as electron donors (6), but which
ones are used by the sulfate-reducing bacteria associated with *A. pompejana* is not clear. Utilization of lactate and pyruvate is almost universal among the sulfate reducers (6), and many species that oxidize energy sources incompletely to excreted acetate can utilize malate, formate, and certain primary alcohols. Those capable of complete oxidation can oxidize electron donors, such as fatty acids, lactate, succinate, and benzoate. These compounds are end products of the hydrolysis and fermentation of complex polymeric compounds which are likely produced by other members of the microbial community utili-
lizing materials produced by the worm (e.g., mucus, hair-like projections, and the dwelling tube are all likely candidates).

In addition to heterotrophic growth on the by-products of fermentation, certain sulfate-reducing bacteria are capable of autotrophic growth with CO₂ as the sole carbon source, H₂ as the electron donor, and sulfate as the electron acceptor. Carbon fixation by bacteria associated with Alvinella would be necessary if grazing by the worm on these bacteria is to make a net contribution to its nutrition (2). There is some evidence of bicarbonate uptake by bacteria associated with A. pompejana (1), but the low level of activity of the carbon-fixing enzyme ribulose bisphosphate carboxylase has cast doubt on the importance of autotrophy (1, 29). Nevertheless, the potential importance of autotrophy in the microbial community associated with Alvinella remains open, because autotrophic sulfate-reducing bacteria fix carbon by using the acetyl-coenzyme A pathway exclusive of ribulose bisphosphate carboxylase (6).

It is unclear which bacterial morphotypes associated with the worm are sulfate reducers. Comparative sequence analysis indicated that the dominant 16S rRNA clone families are aligned with members of the epsilon group of Proteobacteria (18), and in situ hybridization revealed that these phylotypes are of the filamentous morphotype that dominates the community (8). Affiliation of the filaments with the epsilon groups suggests that they may not be sulfate reducers, because no cultivated members of the epsilon group of Proteobacteria are known to reduce sulfate. Sulfate-reducing bacteria come from many taxonomic groups, including the Nitrospira division, the Thermodesulfobacterium division, and the gram-positive group, and there is one archaeal representative, but most are members of the delta group of Proteobacteria (6), a group from which the epsilon subdivision was only recently separated (26). It may be possible to resolve which morphotypes are sulfate reducers without cultivation by using in situ PCR (20, 27) and fluorescence microscopy to localize dissimilatory bisulfite reductase genes within individual cells comprising the community.

There is growing evidence for interactions between bacterial and geological processes at deep-sea hydrothermal vents. The role that Alvinella spp. and their associated bacteria play in altering the growth and morphology of sulfide chimneys is not clear, but a strong influence is indicated. Where colonies of Alvinella spp. occur on the East Pacific Rise, there exist particular morphological types of chimneys known as white smokers or snowball diffusers which are colonized by the Alvinella spp. Elsewhere on the East Pacific Rise, where Alvinella spp. are absent, these chimney morphologies are absent as well, even though the chemistries of the vent fluids are similar (7, 28, 30). It has been postulated that sulfate-reducing bacteria associated with Alvinella spp. might play a role in the physical cementing of Alvinella dwelling tubes to smoker rocks (5).

Understanding the interaction between A. pompejana and its associated microbes requires identification of the abundant members of the community and the major metabolic capacities present. In this study, we established that at least two phylogenetic groups of previously uncharacterized dissimilatory sulfate reducers are present in the community. The impact of sulfate-reducing members of the community and their interaction with the worm remain to be determined. Links with polymer-hydrolyzing and fermentative members of the community and an autotrophic role for these sulfate reducers are anticipated.

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